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Differentiation of Eastern North American *Athyrium filix-femina* Taxa: Evidence From Allozymes and Spores

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ABSTRACT.—*Athyrium filix-femina* (Lady Fern) comprises a complex of homoploid (n = 40) taxa, distributed over much of the northern hemisphere and extending into South America, whose evolutionary relationships are poorly understood and whose taxonomic treatment is problematic. The *A. filix-femina* complex of North America comprises as many as four taxa with overlapping ranges and provides an especially suitable context for exploring patterns and processes of divergent evolution and its taxonomic consequences in ferns. We addressed differentiation of two eastern North American taxa distinguished on the basis of growth form, frond shape, and spore color, and most recently treated as varieties *A. angustum* and *A. asplenioides* (Northern and Southern Lady Fern respectively). Although, these two taxa have been long perceived as closely related, they have been known to intergrade and recombine to form a hybrid zone in their relatively narrow region of overlap. This perception is supported by the data from the present study. Collections from 17 populations, 9 of *A. angustum* from Quebec to Pennsylvania and 8 of *A. asplenioides* from New Jersey to North Carolina, were examined for spores using LM, SEM, and TEM, and/or allozymes (16 loci coding 10 enzymes). The two taxa exhibited highly distinct perispore surfaces: all *A. angustum* individuals had papillose surfaces, whereas most *A. asplenioides* individuals were rugose with a reticulum of inflated folds. Spores from the northernmost *A. asplenioides* population sampled (Shirley, NJ) showed varying degrees of intermediacy suggestive of introgressive hybridization with *A. angustum*. Levels of allozyme polymorphism in populations (means: P=36.5%, A=1.97, H_E=0.129) were near means for angiosperms and ferns. Genotype frequencies at most loci in all populations were in Hardy-Weinberg equilibrium indicating an outcrossing mating system. Most alleles were shared among all populations. However, at the four most polymorphic loci (*Idh-1*, *Pgi-2*, *Pgm-2*, and *Tpi-2*) allele frequencies were significantly divergent between populations of *A. angustum* and *A. asplenioides*, especially *Idh-1* which approached fixation for alternate alleles. Values for F_{ST} ranged from 0.008 to 0.459 for individual loci (0.255 across loci) with especially high values for *Idh-1*, *Pgi-2*, *Pgm-2*, and *Tpi-2*. Hierarchical F_{ST} analysis indicated that differences between the two taxa (F_{XY}=0.216) accounted for most allele frequency divergence among populations (F_{XY}=0.238). UPGMA analysis of paired Rogers' Similarity (S) values resulted in two principal clusters each comprising populations of one taxon. Populations of *A. angustum* and *A. asplenioides* were joined within their clusters at S=0.938 and S=0.945 respectively, while the two taxon clusters were joined at S=0.848. The spore and isozyme data indicate substantial divergence between *A. angustum* and *A. asplenioides*, suggesting that they merit distinction at the rank of subspecies or species. Additional study of populations in their region of sympatry is required to determine the nature and extent of hybridization.

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Athyrium filix-femina (L.) Roth (Lady Fern) comprises a wide-ranging complex of divergent, homoploid (n = 40) taxa of allopatric or parapatric distribution, variously divided and treated at different ranks (species, subspecies, or variety) by different authors. At least three taxa occur in North America, which Butters (1917) treated as separate species "amply distinct from each other," although he considered *A. filix-femina* from western North America as conspecific with the "true" *A. filix-femina* of Europe. Wherry (1961) followed Butters (1917) in recognizing as distinct species the western *A. filix-femina* (specifying var. *sitchense* Ruprecht), the northeastern *A. angustum* (Willd.) Presl., and the southeastern *A. asplenioides* (Michx.) A. Eaton, although noting that they "intergrade to such an extent as to defy any simple classification." Lellinger (1985) treated these taxa as subspecies of a single globally distributed species, i.e., western *A. filix-femina* ssp. *cyclosorum* (Rupr.) C. Chr., northeastern *A. filix-femina* ssp. *angustum* (Willd.) Clausen and the southeastern *A. filix-femina* ssp. *asplenioides* (Michx.) Hultén. More recently, Kato (1993), stating that "the delimitation and infraspecific classification of *A. filix-femina* need detailed study," treated these taxa at varietal rank, recognizing four North American varieties: in the west, northern *A. filix-femina* var. *cyclosorum* Ruprecht (= var. *sitchense*), a more southerly *A. filix-femina* var. *californicum* Butters (which Butters, 1917 had noted as distinctive but treated as a variety of *A. filix-femina*), and in the east, northern *A. filix-femina* var. *angustum* (Willd.) G. Lawson, as well as a southern *A. filix-femina* var. *asplenioides* (Michx.) Farwell.

These differences in classification reflect neither strongly divergent viewpoints among authors as to the nature of species versus infraspecific taxa, nor differing insights following acquisition of compelling data. Rather, they reflect the best judgment of individual authors in the face of a lack of critical information as to character consistency, degree of intergradation, and genetic relationships among taxa in a group noted for a high degree of variability (Schneller and Schmid, 1982). As a contribution toward elucidating the nature of the taxa composing the *A. filix-femina* complex, the present investigation addressed the distinctness of the two parapatric taxa of eastern North America, *A. filix-femina* var. *angustum* and *A. filix-femina* var. *asplenioides* (referred to henceforth informally as bare epithets *angustum* and *asplenioides* respectively). Characters differentiating these taxa, provided by Butters (1917) and by and large echoed in more recent treatments (Lellinger, 1985; Kato, 1993), include rhizome habit, leaf shape, and notably color and surface features of the spores (Table 1). The goal of the present study was to evaluate more fully the degree to which these putative taxa are distinct by 1) characterizing fine features of the spores using electron microscopy, and 2) surveying allozyme variation across a north-to-south transect through the eastern portion of the range of both taxa.

Materials and Methods

Collections.—All observations were made on plants collected by CLK and CRW from 17 localities, nine *A. angustum* and eight *A. asplenioides* (Table 2).

TABLE 1. Comparison of morphological features of the two putative eastern North American taxa in the *Athyrium filix-femina* complex. Compiled from taxon descriptions in Butters (1917), Lellinger (1985), and Kato (1993).

Character	*A. angustum*	*A. asplenioides*
Rhizome orientation	erect or ascending, more condensed	ascending to creeping, more extended
Scales:	linear lanceolate, 8–10 × 1.5–2 mm, brown to dark brown	lanceolate, 3–9 × 2–3 mm, bronze to light brown or brown
Stipe length relative to lamina	up to half the lamina length	equaling lamina length
Lamina shape	elliptic or rhombic	narrowly deltoid lanceolate, ovate-lanceolate to lanceolate
Frond base	gradually tapered to an acute to obtuse base	slightly reduced and truncate at base
Frond apex	acute to acuminate	acute, acuminate, or more-or-less caudate
Widest portion of lamina	near or just below middle	second pinna pair
Pinna attachment	short-stalked or sessile	usually stalked
Pinna shape	oblong-lanceolate, usually widest at middle and not parallel-sided	oblong-lanceolate to lanceolate, nearly parallel-sided
Pinna apex	acute to acuminate	acute
Sterile v. fertile frond	tending toward dimorphism, the segments of fertile fronds narrower and more acute than those of the sterile fronds	not tending toward dimorphism
Pinnules of fertile fronds	narrowly lanceolate and acute	oblong or linear-oblong and obtuse
Indusium length	tending to be shorter than in *asplenioides*, up to 1.1 mm	tending to be longer than in *angustum*, up to 1.3 mm
Indusium margin[a]	irregularly dentate, and/or ciliate with eglandular hairs	ciliate with glandular or non-glandular hairs as long as indusial width
Sporangial stalks	bearing glandular hairs or less often secondary sporangia	consistently bearing glandular hairs
Spore color	yellowish	brownish-yellow to dark-brown or black
Spore surface	sparsely papillate	wrinkled or reticulated exospore, sometimes nigrescent
Mean spore dimensions	38.6 × 24.7 μ	36.0 × 25.5 μ

[a] Descriptions of the indusium margin in the two taxa are inconsistent in the literature. The table entry is a tentative consensus.

TABLE 2. Collection localities for *Athyrium filix-femina* populations examined in this study. Localities are ordered from north to south.

Population Designation	Collector(s) and collection number	Year Collected	Locality	No. of isozyme samples	No. of spore samples
	A. filix-femina* var. *angustum				
Mt. Sainte Hilaire	*C. Werth s. n.*	1997	Province du Quebec, along hiking trail through forest, Mont Ste.-Hilaire	26	7
Mast Landing	*L. B. Kass s. n.*	1987	Maine, Cumberland County, Mast Landing Sanctuary, Freeport	0	4
North Hudson	*C. Werth and C. Caplen s. n.*	1997	New York, Essex County, *Abies/Thuja* woods and stream bank, 11.3 mi W of North Hudson and I-87, exit 387 to Blue Ridge	26	10
Schenevus	*C. Werth and C. Caplen s. n.*	1997	New York, Otsego County, woods near edge E side I-88, near Schenevus	11	1
Barnet	*C. Werth, D. Conant, and C. Caplen s. n.*	1997	Vermont, Caledonia County, ditches and woods edge along dirt road in vicinity of Conant farm, near Barnet	72	20
Ithaca	*CL Kelloff 560*	1988	New York, Tompkins County, fen along Rt. 96 south of junction with Route 13 approximately 2 miles south of Ithaca	0	20
Binghamton	*CL Kelloff 559*	1988	New York, Broome County, floodplain of Chenango R., 1.5 miles N of Binghamton	0	20
Ralston-1	*CL Kelloff 569*	1988 & 1995	Pennsylvania, Lycoming County, flood plain along Route 14, 7 miles north of Ralston	20	58
Ralston-2[1]	*CL Kelloff 570*	1988	Pennsylvania, Lycoming County, flood plain along Route 14, 3 miles south of Ralston	0*	78

TABLE 2. Continued.

Population Designation	Collector(s) and collection number	Year Collected	Locality	No. of isozyme samples	No. of spore samples
	A. filix-femina* var. *asplenioides				
Shirley	*CL Kelloff 1289*	1997	New Jersey, Elmer County, floodplain along County Road 611, 1.5 miles east of jct. NJ State Route 77, in Shirley	40	27
Breathed Mountain	*CL Kelloff 25*	1987	West Virginia, Tucker County, Breathed Mountains	0	6
George Mason	*CL Kelloff 101*	1988	Virginia, Fairfax County, creekside on George Mason University campus in Fairfax	0*	30
Hopewell	*CL Kelloff 565*	1988 & 1995	Virginia, Fauquier County, floodplain along Route 629, 3.3 miles N of Route 601 near Hopewell	30	42
Mountjoy Store	*CL Kelloff 564*	1988 & 1995	Virginia, Stafford County, bog at Mountjoy Store, junction of routes 611 and 633	30	63
Pond Drain	*C. Werth, E. Potter, and K. Sciaretta s. n.*	1997 & 1998	Virginia, Giles County, north-facing wooded slope along rd. to White Pine Lodge and above Pond Drain, NW of rt. 613 and Mountain Lake	50	0
Pipevine Hollow	*CL Kelloff s. n.*	1987	Tennessee, Davidson County, Pipevine Hollow	0	**?5**
Sandy Run Swamp	*CL Kelloff 1285*	1997	North Carolina, Onslow County, creek bank and roadside ditch E side of Sandy Run Swamp along Haws Run Road, just W of Padgett, ca. 60 air km NE of Wilmington	32	18

[1] Initial isozyme data were reported for 78 individuals from Ralston-2 (Kelloff 1990), but this population was not resampled during 1995 and isozyme data are not included in the present report (see text for further explanation).

Of these, seven were examined only for spores, while for ten (five of each taxon), population samples for isozyme electrophoresis were obtained as well. At most localities, plants were collected in a transect and individuals numbered successively. Voucher specimens for localities collected by CLK are deposited at the George Mason University Herbarium (GMUF), those collected by CRW at the E. L. Reed Herbarium of Texas Tech University (TTC).

Spore Morphology.—Spores were collected from fresh samples gathered from 16 of the localities listed in Table 2. The spores were examined for surface characteristics of the perispore using light microscopy (LM; 409 individuals), and scanning electron microscopy (SEM; one to two individuals from ten populations, 13 from the Shirley, NJ population). For exine ultrastructure, transmission electron microscopy (TEM) was used to examine four individuals, 2 of each taxon. For LM, spores were mounted by gently tapping a pinnule bearing mature sporangia over a glass slide and then adding a few drops of Permount™ mounting medium and a glass coverslip. Spores were examined under a standard brightfield optic system.

For SEM, air dried spores were affixed to glass coverslips with ethanol, mounted on SEM stubs, and sputter coated with gold or carbon/gold palladium using the Hummer VII sputtering system. The spores were then examined using either a Hitachi S-530 or a LEO 440 scanning electron microscope. From each SEM sample at least 100 spores were examined.

For TEM, each spore sample was embedded in 1% agar as a pellet and fixed in sodium veronal-acetate buffered potassium permanganate (Dawes, 1971). The sample pellet, cut up into 1.0 mm cubes, was dehydrated in a graded series of ethanol. After dehydration, the spores were infiltrated with a series of propylene oxide and Spurrs plastic embedding medium in a labeled BEEM capsule and polymerized in an oven at 70°C for 16 hours. The blocks were then thin-sectioned with a Dupont diamond knife, the sections picked up on #200 mesh grids and examined with a JEOL 100C transmisson electron microscope. Post-staining of the spore sections was not necessary when fixed with the buffered potassium permanganate (Dawes, 1971).

Isozyme Electrophoresis.—Fronds to be analyzed electrophoretically were collected in the field and kept refrigerated in plastic bags until homogenized within a few days. For each sample, approximately 1 g leaf tissue was ground using eight drops of "microbuffer" (Werth, 1985) enhanced with 5% PVP-40T and 1% 2-mercaptoethanol. The tissue was ground in a porcelain spot plate using a small test tube as a pestle and sand to facilitate grinding. The extract was either absorbed onto filter paper wicks and used immediately or stored at −78°C for up to four months before being thawed and absorbed onto wicks. Starch gel concentration varied from 11% to 13.5% depending on the properties of individual starch lots.

The following eleven enzymes were analyzed using the buffer systems indicated: on lithium hydroxide (Selander *et al.*, 1971)—glutamate oxaloacetate transaminase (GOT), hexokinase (HK), and leucine aminopeptidase (LAP); on system number 6 (Soltis *et al.*, 1983)—phosphoglucose isomerase

(PGI), phosphoglucomutase (PGM), and triose-phosphate isomerase (TPI); on morpholine-citrate pH 8.2 (Werth, 1991)—aldolase (ALD), isocitrate dehydrogenase (IDH), malate dehydrogenase (MDH), 6-phosphogluconate dehydrogenase (6PGDH), and shikimate dehydrogenase (SKDH). Gels were run until the bromophenol blue marker migrated 10 cm for lithium-hydroxide and number 6, or 12 cm for morpholine-citrate. Staining followed Werth (1985) as modified for the zymecicle technique (Werth, 1990). Isozyme banding patterns were readily interpreted as allelic variation for single gene loci from whole leaf extracts (cf. Gastony and Darrow, 1983) using standard principles (Wendel and Weeden, 1989; Murphy *et al.*, 1990). Data were analyzed using the BIOSYS-1 computer program (Swofford and Selander, 1981), entered into the program as individual multilocus genotypes. Occasionally, sets of two or three successive samples from the field possessed identical genotypes. As *Athyrium filix-femina* is known to occasionally form clones up to nearly 20 m in extent (Sciaretta *et al.*, ms), such sets were assumed to comprise single genets and each set was represented by only a single entry in the data set.

Initial isozyme data were obtained from four populations, two of each taxon (data reported in Kelloff, 1990). During 1995, three of these populations (Ralston-1, PA, Hopewell, VA, and Mountjoy Store, VA) were revisited and re-sampled, and an expanded isozyme data set obtained; this was augmented by sampling of additional populations during 1997 and 1998. The initial data were largely consistent with the more recent results; however, only the latter are reported here to avoid including inadvertently re-sampled individuals.

Results

Spores, Light Microscopy.—Observation of spores through the light microscope (LM) revealed features largely consistent with previous reports of differences between the two taxa (Butters, 1917; Lugardon, 1971; Schneller, 1989). Spores of both *A. angustum* (Fig. 2a, b) and *A. asplenioides* (Fig. 3a, b) were monolete with an unbranched longitudinal proximal laesura, ovoid to ellipsoidal in polar view, and possessed a perispore (terminology of Erdtman, 1969). Although most of the surface details were obscure when viewed under LM, the difference between the two taxa was readily seen in their outline. The spores of *A. angustum* had a somewhat smooth surface (Fig. 2a, b), while those of *A. asplenioides* had an uneven surface (Fig. 3a, b).

Spore color has been described as differing between *A. angustum* with yellowish spores, and *A. asplenioides* with yellowish-brown to blackish spores (Table 1). This characterization evidently is based on appearance of mass spores under reflected light as viewed unaided or with a dissecting microscope. When viewed under LM using transmitted light, spores of both taxa appeared yellowish, with the exception of occasional *A. asplenioides* spores that displayed an especially dark or blackish reticulation overlaying the yellow surface of the spore.

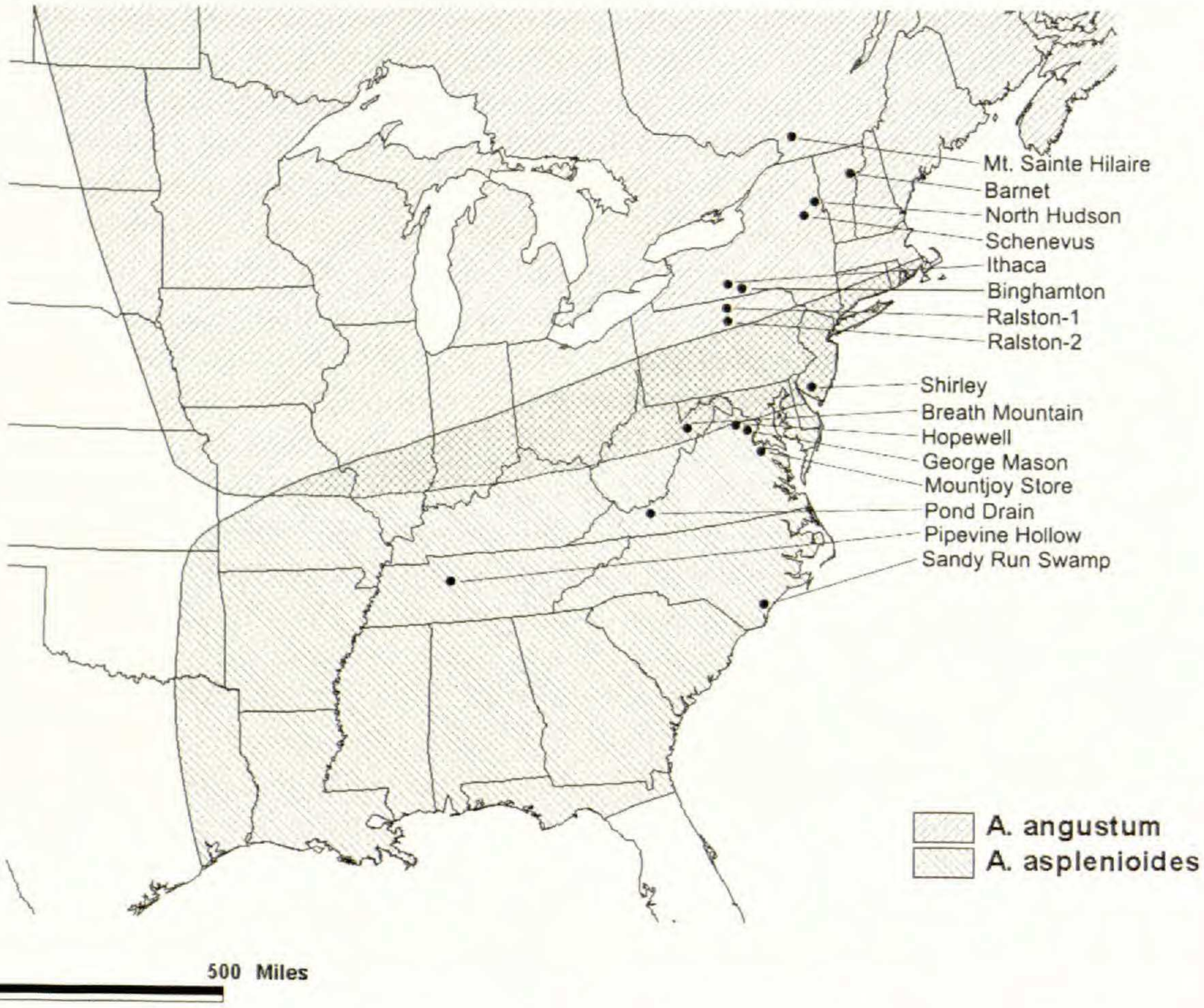

FIG. 1. Map showing overlapping ranges of two *Athyrium* taxa, *angustum* and *asplenioides*, in eastern North America and location of populations studied.

SCANNING ELECTRON MICROSCOPY.—Observations of spores under SEM were consistent with those of Tryon and Lugardon (1991) and Schneller (1989) in revealing that the perispore of *A. angustum* (Fig. 2c–f) differs markedly from that of *A. asplenioides* (Fig. 3c–f). The perispore of *A. angustum* was papillose, its surface completely and evenly covered with minute verruciform (i.e., wart-like) projections. On some spores of *A. angustum*, thin, irregular laminae of perispore material appeared as occasional "flakes." In contrast, the perispore of *A. asplenioides* was rugose, characterized by a network of muri (i.e. ridges/walls) that meandered and joined to surround lacunae. The surface of the lacunae completely lacked the projections as in *A. angustum*.

TRANSMISSION ELECTRON MICROSCOPY.—Under TEM the similarities and differences between the structural and sculptural features of the spore wall of the two taxa were clearly distinct. Both *A. angustum* and *A. asplenioides* (Figs. 2f and 3f) possessed a thick external exospore (Ee). Although not evident in these sections, the fern exospore is composed of feuillettes, i.e., fused plates (Lugardon, 1976, 1979; Tryon, 1986). TEM revealed that the sculptural elements composing the surface details seen under SEM were not derived from

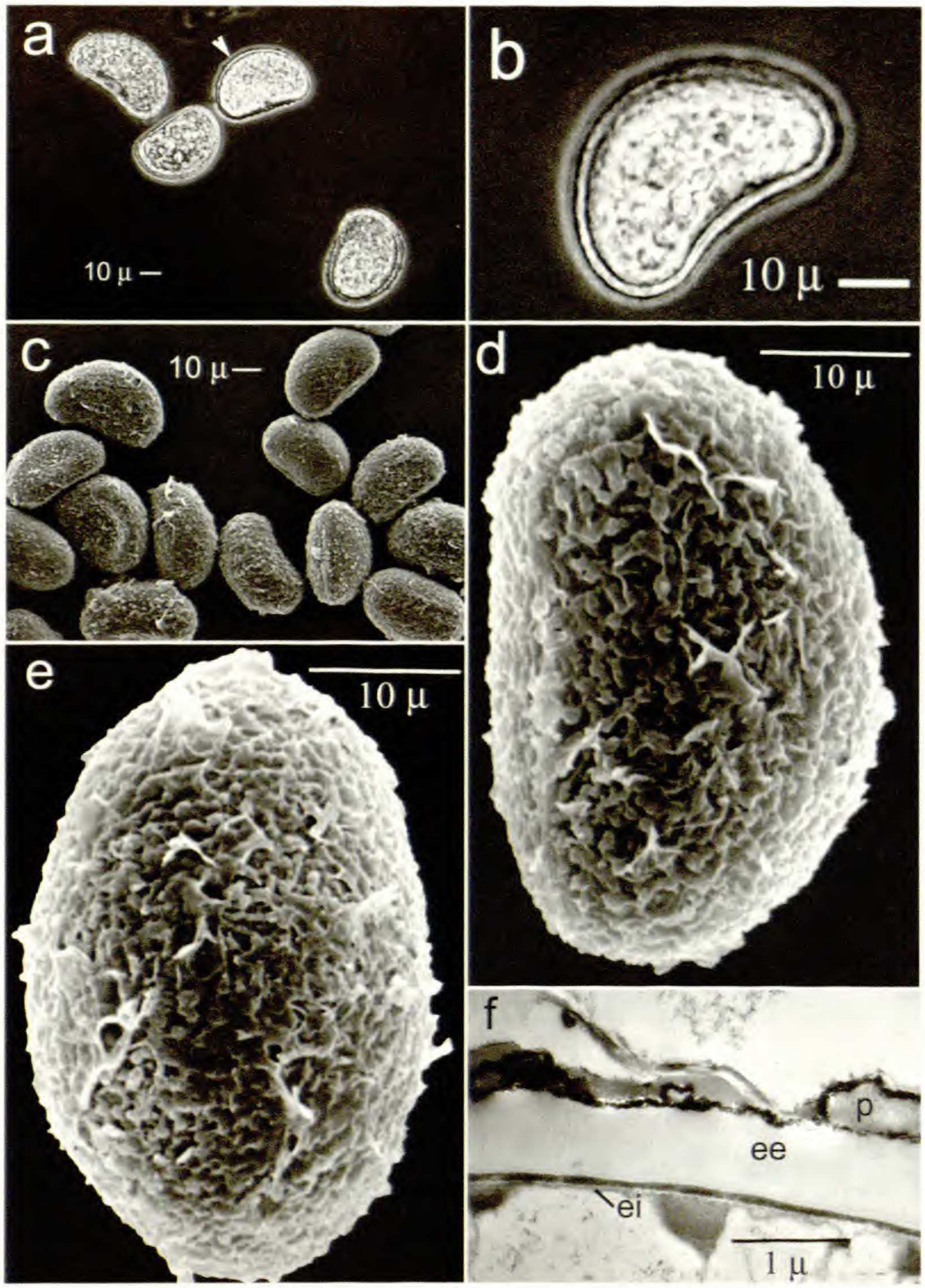

FIG. 2. Spores of *Athyrium angustum*. A, B. Spores viewed under LM. Note smooth appearance of perispore. C, D, E. Spores viewed under SEM. Note papillate perispore surface. F. Spore wall viewed under TEM. Note low perispore (p), external exospore (ee), and internal exospore (ei).

the exospore, as for example in the spores of *Trichomanes* or *Protomarattia* (Tryon, 1986), but instead from the perispore, the outer stratum circumjacent to the external exospore. The perispore of both taxa fit tightly to the external exospore. This stratum in the spores of *A. angustum* (Fig. 2f) was a shallow,

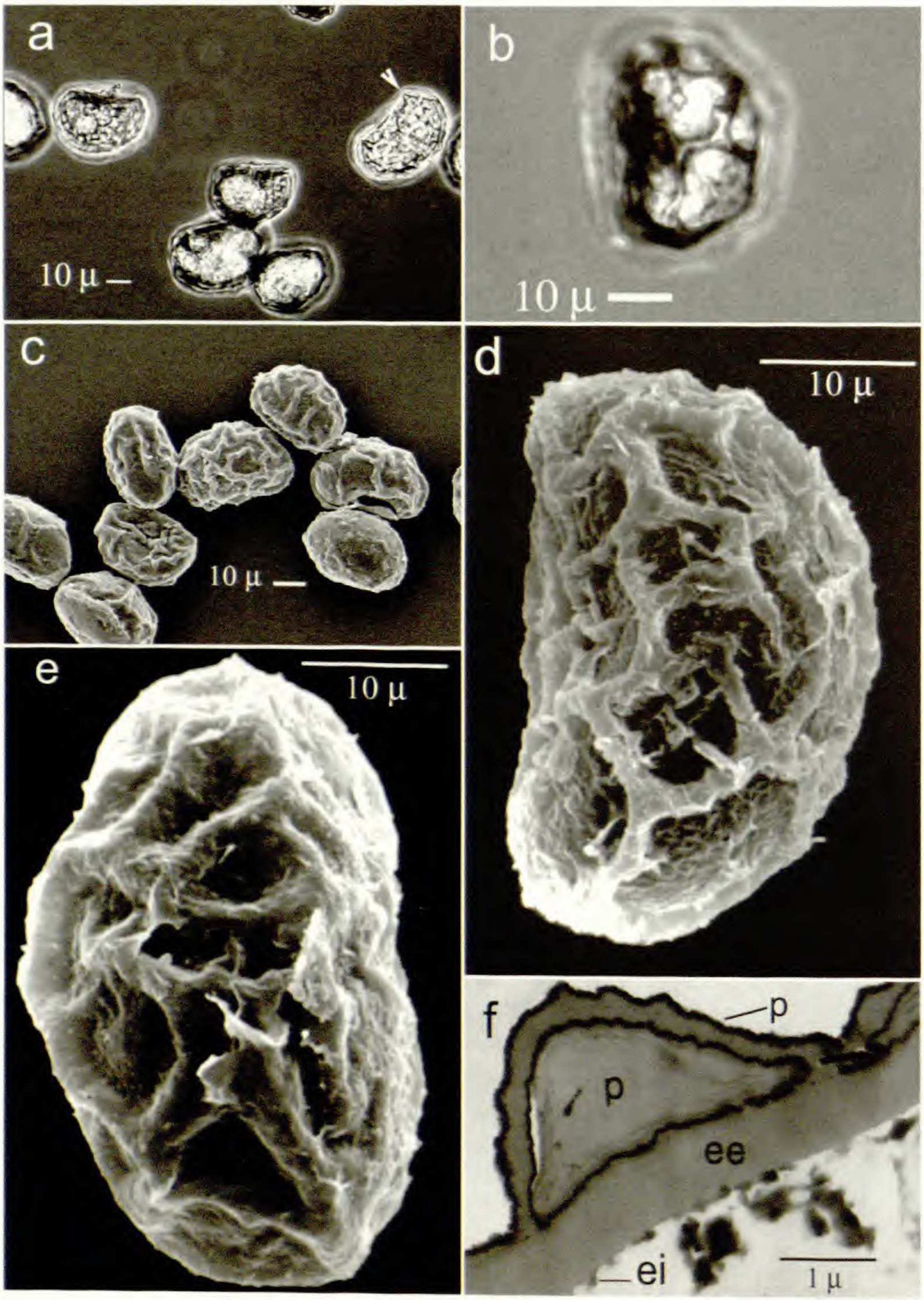

FIG. 3. Spores of *Athyrium asplenioides*. A, B. Spores viewed under LM. Note rugose, reticulate appearance of perispore. C, D, E. Spores viewed under SEM. Note reticulating inflated folds of perispore surface. F. Spore wall viewed under TEM. Note inflated fold in perispore (p), external exospore (ee), and faint internal exospore (ei).

fairly uniform layer. In *A. asplenioides* spores, what appeared to be muri under the SEM (Figs. 3c–e) were more clearly seen under TEM to be inflated folds in the perispore because the columellae, characteristic of true muri, were lacking (Fig. 3f). Beneath the external exospore on *A. angustum* was

a thin endospore or internal exospore (Ei), a layer that is deposited shortly before germination of the spore (Tryon, 1986). A remnant of this layer was also visible in *A. asplenioides*.

TAXON FIDELITY OF SPORE MORPHOTYPES AND EVIDENCE FOR HYBRIDIZATION.—All populations of *A. angustum* sampled for this study exhibited the smooth, micropapillate spore morphotype, and all populations of *A. asplenioides* except one exhibited the rough, rugose spore morphotype. Reliance on light microscopy, which may fail to reveal the subtle detail of the perispore, and on color, which varies in *A. asplenioides* despite the overall similarity of perispore in this taxon, may have led to some confusion in the previous literature. For example, Liew (1971) concluded that most individuals of both *A. angustum* and *A. asplenioides* produced both kinds of spores, entirely inconsistent with our observations.

The exceptional *A. asplenioides* population was the northernmost population sampled, Shirley, NJ. At this site the spores had perispore sculpturing characteristics of both *A. angustum* and *A. asplenioides*. While none of the plants from this site possessed *A. angustum*-type spores, spore arrays of the 13 plants examined under SEM possessed unique and variable morphologies that suggested an influence from *A. angustum* (Fig. 4). The greatest degree of *A. angustum* influence was exhibited by six individuals in which prevailing perispore morphotype was very irregular in appearance, combining an array of sculptural elements that were difficult to characterize (Fig. 4a, b). These included irregularly shaped papillae, briefly-extending isolated muri, and a preponderance of irregular small lamellae reminiscent of the "flakes" seen in *A. angustum*. This prevailing morphotype is readily interpreted as intermediate between the highly distinct morphotypes of *A. angustum* and *A. asplenioides*, seeming to average their disparate surface features and indicating that these individuals may be hybrids between the two taxa. Many of the highly variable spores of these putative F1 hybrids were not entirely intermediate, but rather showed tendencies toward either the smooth *A. angustum* type or the rough *A. asplenioides* type (Fig. 4a, c–g). Spores that fully resembled parental types were discovered in the spore arrays of these six individuals, although these extremes were decidedly rare. The spores of these putative hybrids were not abortive, as is typically the case in interspecific fern hybrids, but rather appeared normal, i.e., well-filled, and were found to be viable as evidenced by successful germination (unpublished data). Moreover, the seven other individuals in the Shirley, NJ, population examined under SEM possessed spore arrays exhibiting various degrees of intermediacy but strongly skewed toward the *A. asplenioides* morphology. These are hypothesized to include backcrosses between first-generation hybrids and *A. asplenioides* (two individuals) and later generation backcrosses (five individuals).

ALLOZYMES, GENERAL.—The eleven enzymes assayed were coded by 17 interpretable loci of which only one (*Pgi-1*) was invariant across all ten populations. The remaining sixteen loci (*Ald, Got, Hk, Idh-1, Lap, Mdh-1, Mdh-2,*

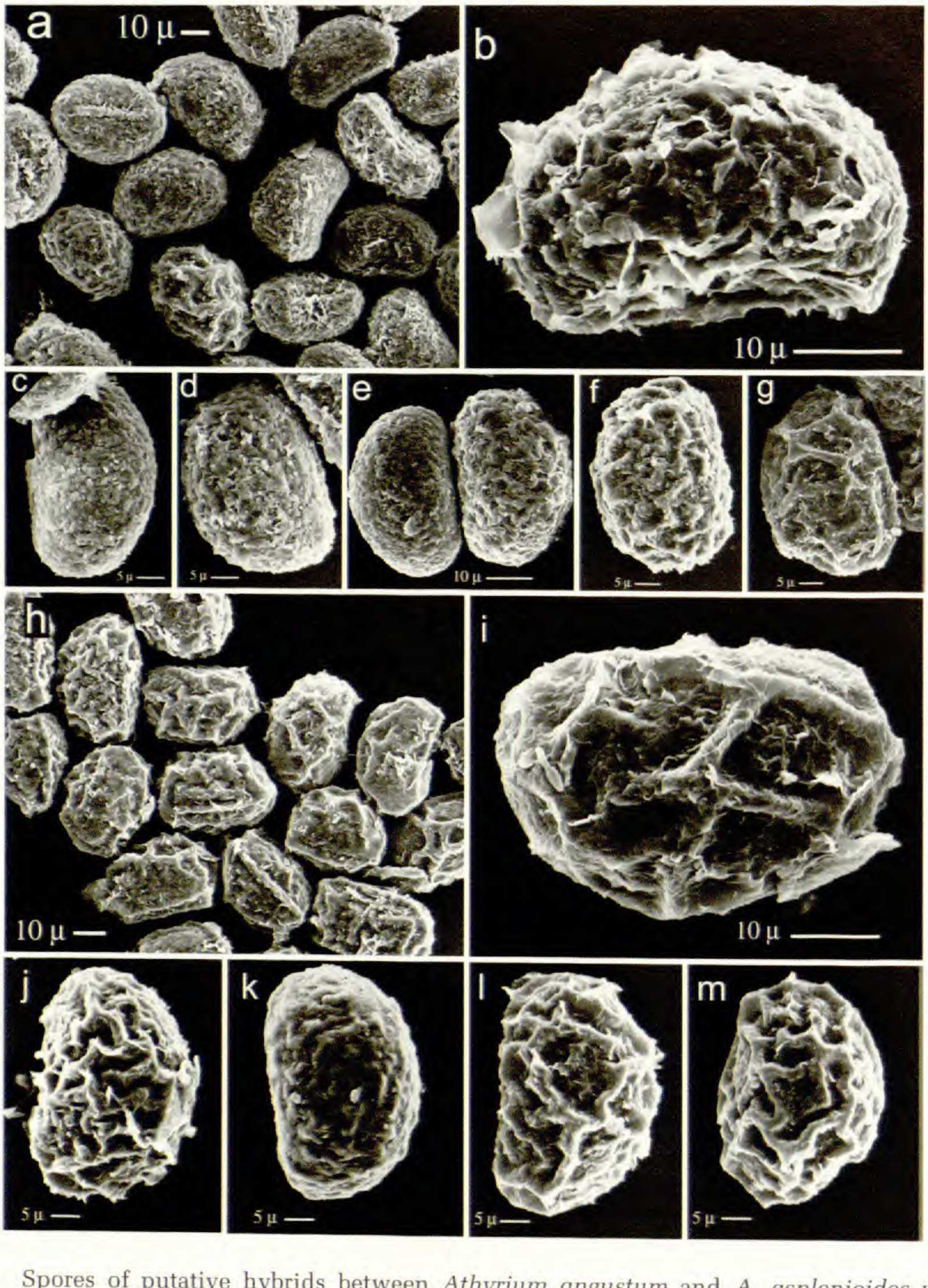

FIG. 4. Spores of putative hybrids between *Athyrium angustum* and *A. asplenioides* viewed under SEM. A–G, spores of putative first-generation hybrid. A. Array of spores. Note variable morphology and preponderance of "flaky" intermediates. B. Close-up of "flaky" intermediate spore morphotype. C–G. Examples of spore morphotypes occurring in putative F-1 hybrid, varying from *A. angustum*-like (C) to *A. asplenioides*-like (G). H–M, spores of putative backcross between F-1 hybrid and *A. asplenioides*. H. Array of spore morphotypes exhibited by the putative backcross. Note prevalent of *A. asplenioides*-like spores. I. Close-up of *A. asplenioides*-like spore from backcross. Note irresolute nature of inflated folds and tendency toward "flakiness". J–M. Examples of spore morphotypes occurring in putative backcross. Note variation from "flaky" intermediate (J) to *A. asplenioides*-like (M).

Mdh-3, Mdh-4, Pgi-2, Pgm-2, 6-Pgd-1, 6Pgd-2, Skdh, Tpi-1, and *Tpi-2*) were variable in at least one population, and the frequencies of the alleles were computed (Table 3). There was a strong tendency for all populations to share the more common alleles and some of the less frequent ones as well. Most of the loci were weakly polymorphic, a single allele predominating in all populations of both *A. angustum* and *A. asplenioides*. In contrast, at the four most polymorphic loci (*Idh-1, Pgi-2, Pgm-2*, and *Tpi-2*), population allele frequencies were similar within *A. angustum* and *A. asplenioides* respectively, but strikingly different between the two taxa (Table 3).

The most contrasting locus was *Idh-1* for which alleles $Idh\text{-}1^B$ and $Idh\text{-}1^C$ prevailed in *A. angustum* at frequencies ranging from 0.545 to 0.676 and 0.296 to 0.455 respectively, while allele $Idh\text{-}1^A$ was the most frequent in *A. asplenioides* with frequencies ranging from 0.828 to 0.950. At *Pgi-2* both taxa shared $Pgi\text{-}2^E$ as their most frequent allele; however, $Pgi\text{-}2^G$ was represented in all *A. angustum* populations at much higher frequencies, ranging from 0.154 to 0.450, than in populations of *A. asplenioides* for which the frequency of this allele ranged from 0.000 to 0.106. At *Pgm-2*, both taxa shared $Pgm\text{-}2^B$ and $Pgm\text{-}2^C$ as principal alleles, the former prevailing in *A. angustum* populations at frequencies ranging from 0.856 to 1.000, the latter prevailing in *A. asplenioides* populations at frequencies ranging from 0.580 to 0.857. At *Tpi-2* both taxa shared three prevalent alleles, with $Tpi\text{-}2^A$ being of substantial frequency in all populations. Allele $Tpi\text{-}2^B$ occurred at high frequencies, ranging from 0.355 to 0. 550, in *A. angustum* populations whereas $Tpi\text{-}2^C$ was infrequent in this taxon, occurring at frequencies from 0.000 to 0.100. Conversely, $Tpi\text{-}2^C$ often was the most frequent allele in *A. asplenioides* populations, occurring at frequencies from 0.316 to 0.606, while $Tpi\text{-}2^B$ was rarer, occurring at frequencies from 0.000 to 0.255. The contrasting allele frequency trends for these four loci were highly consistent among populations within each taxon. Exceptions to these frequencies trends were in the northernmost sampled *A. asplenioides* population, Shirley, from southern New Jersey for the characteristically *A. angustum* alleles $Idh\text{-}1^B$ and $Pgi\text{-}2^G$ and likewise for $Tpi\text{-}2^B$ in the highest elevation (1300 m) population of *A. asplenioides* sampled, Pond Drain, from the mountains of southwestern Virginia. Moreover, five of the six Shirley, NJ, individuals hypothesized to be first-generation hybrids on the basis of spore morphology were heterozygous for *A. angustum* and *A. asplenioides* marker alleles for at least three of the four most divergent loci, i.e., $Idh\text{-}1^{AB}$, $Pgi\text{-}2^{EG}$, $Pgm\text{-}2^{BC}$ (scored for only two of these individuals) and $Tpi\text{-}2^{BC}$. No other individuals in the entire data set possessed this genotype combination.

GENETIC VARIATION.—Genetic variation was quantified for each population and the species as a whole by computing three standardly used indices. Values for percent loci polymorphic (P) ranged from 23.5% to 47.1%, with a mean of 36.48%; for mean number of alleles per locus (A), the range was 1.5 to 2.5, mean 1.97; and for mean expected heterozygosity (H_E), the range was 0.112 to 0.147, mean 0.129 (Table 4). The mean values for these indices in

TABLE 3. Allele frequencies for 17 isozyme loci in ten populations of *Athyrium filix-femina* in eastern North America.

		angustum					*asplenioides*				
Locus	Allele	Mt. Ste. Hilaire QE	Barnet VT	North Hudson NY	Schenevus NY	Ralston PA	Shirley NJ	Mountjoy Store VA	Hopewell VA	Sandy Run Swamp NC	Pond Drain VA
Ald	A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.969
	B	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.021
	C	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.010
	(N)	19	29	26	11	1	8	1	1	32	48
Got	A	0.000	0.033	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	B	1.000	0.951	1.000	1.000	1.000	0.955	1.000	0.917	1.000	0.980
	C	0.000	0.016	0.000	0.000	0.000	0.045	0.000	0.083	0.000	0.010
	D	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.010
	(N)	10	61	5	6	20	33	30	30	12	50
*Hk**	A	0.000	0.000	0.000	0.000	0.150	0.000	0.017	0.000	0.000	0.016
	B	0.960	0.965	1.000	0.955	0.800	0.868	0.967	0.967	0.893	0.952
	C	0.040	0.023	0.000	0.045	0.050	0.105	0.017	0.017	0.089	0.032
	E	0.000	0.012	0.000	0.000	0.000	0.025	0.000	0.017	0.000	0.000
	F	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.018	0.000
	(N)	25	43	19	11	10	19	30	30	28	31
*Idh-1**	A	0.019	0.007	0.000	0.000	0.000	0.828	0.950	0.833	0.859	0.920
	B	0.596	0.676	0.596	0.545	0.650	0.172	0.017	0.133	0.094	0.060
	C	0.385	0.296	0.385	0.455	0.350	0.000	0.000	0.000	0.000	0.020
	D	0.000	0.021	0.019	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	F	0.000	0.000	0.000	0.000	0.000	0.000	0.033	0.033	0.047	0.000
	(N)	26	71	26	11	20	32	30	30	32	50
Lap	A	0.000	0.000	0.000	-	0.000	0.000	0.018	0.000	0.000	0.000
	B	0.100	0.038	0.063	-	0.175	0.000	0.036	0.000	0.031	0.016
	C	0.850	0.885	0.750	-	0.550	1.000	0.946	1.000	0.938	0.935
	D	0.050	0.058	0.188	-	0.275	0.000	0.000	0.000	0.016	0.032
	E	0.000	0.019	0.000	-	0.000	0.000	0.000	0.000	0.016	0.000
	F	0.000	0.000	0.000	-	0.000	0.000	0.000	0.000	0.000	0.016
	(N)	10	26	16	0	20	33	28	23	32	31

TABLE 3. Continued.

		angustum					*asplenioides*				
Locus	Allele	Mt. Ste. Hilaire QE	Barnet VT	North Hudson NY	Schenevus NY	Ralston PA	Shirley NJ	Mountjoy Store VA	Hopewell VA	Sandy Run Swamp NC	Pond Drain VA
Mdh-1	A	0.981	0.950	0.942	1.000	1.000	0.939	0.967	1.000	0.969	1.000
	B	0.019	0.020	0.058	0.000	0.000	0.061	0.033	0.000	0.000	0.000
	C	0.000	0.030	0.000	0.000	0.000	0.000	0.000	0.000	0.031	0.000
	(N)	26	50	26	11	20	33	15	20	32	50
Mdh-2	A	1.000	0.990	1.000	1.000	1.000	1.000	0.933	0.950	1.000	1.000
	B	0.000	0.010	0.000	0.000	0.000	0.000	0.067	0.050	0.000	0.000
	(N)	26	50	26	11	20	33	15	20	32	50
Mdh-3	A	1.000	1.000	1.000	1.000	1.000	0.985	0.967	1.000	0.953	1.000
	B	0.000	0.000	0.000	0.000	0.000	0.015	0.033	0.000	0.047	0.000
	(N)	26	50	26	11	20	33	15	20	32	50
Mdh-4	A	0.960	0.920	0.962	1.000	1.000	1.000	0.967	0.950	1.000	1.000
	B	0.040	0.070	0.038	0.000	0.000	0.000	0.033	0.050	0.000	0.000
	C	0.000	0.010	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	(N)	25	50	26	11	13	33	15	20	32	50
Pgi-1	A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	(N)	22	67	21	11	10	13	25	24	27	38
Pgi-2	A	0.000	0.000	0.000	0.000	0.000	0.015	0.033	0.000	0.000	0.000
	B	0.000	0.014	0.019	0.000	0.000	0.000	0.017	0.133	0.000	0.000
	C	0.019	0.007	0.000	0.000	0.000	0.000	0.000	0.017	0.000	0.010
	D	0.000	0.000	0.000	0.000	0.000	0.045	0.100	0.000	0.078	0.010
	E	0.692	0.715	0.808	0.500	0.550	0.742	0.800	0.850	0.906	0.930
	F	0.019	0.014	0.000	0.000	0.000	0.045	0.017	0.000	0.000	0.020
	G	0.269	0.250	0.154	0.409	0.450	0.106	0.033	0.000	0.016	0.030
	H	0.000	0.000	0.000	0.000	0.000	0.045	0.000	0.000	0.000	0.000
	I	0.000	0.000	0.019	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	J	0.000	0.000	0.000	0.091	0.000	0.000	0.000	0.000	0.000	0.000
	(N)	26	72	26	11	20	33	30	30	32	50
Pgm-2	A	0.024	0.000	0.000	0.000	0.000	0.000	0.000	0.083	0.000	0.020
	B	0.857	0.856	0.906	1.000	0.975	0.143	0.300	0.100	0.222	0.390
	C	0.119	0.122	0.094	0.000	0.025	0.857	0.683	0.800	0.704	0.580

TABLE 3. Continued.

		angustum					*asplenioides*				
Locus	Allele	Mt. Ste. Hilaire QE	Barnet VT	North Hudson NY	Schenevus NY	Ralston PA	Shirley NJ	Mountjoy Store VA	Hopewell VA	Sandy Run Swamp NC	Pond Drain VA
	D	0.000	0.022	0.000	0.000	0.000	0.000	0.017	0.017	0.074	0.000
	E	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.010
	(N)	21	45	16	11	20	21	30	30	27	50
6Pgd-1	A	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	B	1.000	1.000	1.000	1.000	1.000	0.957	1.000	1.000	1.000	1.000
	C	0.000	0.000	0.000	0.000	0.000	0.043	0.000	0.000	0.000	0.000
	(N)	11	38	11	6	10	23	14	15	15	44
6Pgd-2	A	0.000	0.000	0.000	0.000	0.000	0.015	0.033	0.017	0.017	0.021
	B	1.000	1.000	1.000	1.000	1.000	0.985	0.950	0.967	0.983	0.947
	C	0.000	0.000	0.000	0.000	0.000	0.000	0.017	0.017	0.000	0.021
	D	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.011
	(N)	26	69	21	11	20	33	30	30	30	47
Skdh	A	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.067	0.000	0.000
	B	0.981	0.955	0.942	0.955	1.000	1.000	1.000	0.933	1.000	0.990
	C	0.019	0.036	0.058	0.045	0.000	0.000	0.000	0.000	0.000	0.010
	D	0.000	0.009	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	(N)	26	56	26	11	20	33	30	30	32	50
Tpi-1	A	0.947	1.000	0.981	0.900	1.000	0.970	1.000	1.000	0.984	1.000
	B	0.053	0.000	0.000	0.100	0.000	0.030	0.000	0.000	0.016	0.000
	C	0.000	0.000	0.019	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	(N)	19	47	26	5	10	33	25	25	32	18
*Tpi-2**	A	0.579	0.601	0.404	0.400	0.450	0.318	0.440	0.420	0.406	0.429
	B	0.368	0.355	0.538	0.500	0.550	0.076	0.100	0.000	0.063	0.255
	C	0.053	0.036	0.058	0.100	0.000	0.606	0.460	0.580	0.531	0.316
	E	0.000	0.007	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	(N)	19	69	26	5	10	33	25	25	32	49

these eastern North American *Athyrium* populations were somewhat greater than the means for ferns (P=36.0%, A=1.65, H=0.109) obtained by averaging across 32 taxa (Li and Haufler, 1999), which are in turn similar to the means for angiosperms (P=34.2%, A=1.53, H_E=0.113; Hamrick and Godt, 1989). Mean values across *A. angustum* populations for P (34.12%) and A (1.84) were slightly lower than those for *A. asplenioides* (mean P=38.84%, mean A=2.1), while the mean value in *A. angustum* for H_E=0.138 was greater than that of 0.119 in *A. asplenioides*. This indicates that while *A. asplenioides* possesses greater allelic diversity, frequencies of alleles within loci are more evenly distributed in *A. angustum* populations.

Values for mean observed heterozygosity (H_O) were also computed for comparison to H_E. Values of H_O were very similar to (although tending to be slightly greater than) those for H_E, suggesting a tendency toward random mating.

COMPARISON TO HARDY-WEINBERG.—Genotype proportions for each polymorphic locus in each population were compared to Hardy-Weinberg expected values by computing the fixation index F, and the statistical difference of F from 0 was evaluated using the chi-square test, with pooled genotype classes if the number of alleles exceeded 2 (Table 5). The test was considered valid if two of the three genotype classes were represented by expected values $\geqslant 5$. Of 42 validly tested loci, 40 conformed to Hardy-Weinberg expectations. Moreover, 45 out of 51 non-valid tests also indicated conformance to Hardy-Weinberg values, despite the tendency for non-valid tests to indicate false non-conformance due to low expected values. Thus, mating was inferred to approximate random mating via predominant outcrossing between gametophytes, as appears to be the general case in most ferns (Soltis *et al.*, 1988) including *Athyrium* species (Schneller, 1979).

GENETIC RELATEDNESS OF POPULATIONS AND TAXA.—The degree of genetic divergence among populations (Table 6) was quantified by computing F-statistics (Wright, 1965, 1978), including hierarchical analysis (Wright, 1978). Values for F_{ST} computed among all populations varied among loci from a low of 0.019 for *6Pgd-2* to high values of 0.468 for *Pgm-2* and 0.460 for *Idh-1*, the latter two loci having exhibited the greatest allele frequency difference between *A. angustum* and *A. asplenioides*. The value across loci of F_{ST}= 0.255 indicated very substantial differentiation among populations. This value is very high in comparison to other fern species examined, exceeding, for example, computed values for mean F_{ST} of 0.024 among populations of *Polystichum munitum* ranging from Oregon to Idaho (Soltis *et al.*, 1987), F_{ST}= 0.152 among *Pteridium aquilinum* populations ranging from Massachusetts to Florida (Speer *et al.*, 1998), and F_{ST}=0.100 to 0.248 in various species of *Dryopteris* ranging widely across eastern North America (Werth, ms.).

To evaluate the contribution of differences between *A. angustum* and *A. asplenioides* to overall population differentiation, hierarchical F-statistic analysis (Wright, 1978) was carried out (Table 6). For most individual loci, as well as for the combined values across loci, the variance between the two

TABLE 4. Estimates of genetic variation at 17 loci in ten populations of *Athyrium filix-femina s. l.* (standard errors in parentheses).

Population	Mean sample size per locus	Mean no. of alleles per locus (A)	Percent loci polymorphic[a] (P)	Mean heterozygosity	
				Observed (H_o)	Hardy-Weinberg Expected (H_E)
A. angustum					
Mt. Ste. Hilaire, QE	21.4 (1.4)	1.9 (0.2)	35.3	0.150 (0.056)	0.140 (0.047)
Barnet, VT	52.5 (3.4)	2.5 (0.3)	41.2	0.134 (0.042)	0.141 (0.042)
North Hudson, NY	21.7 (1.5)	1.8 (0.2)	41.2	0.133 (0.046)	0.136 (0.047)
Schenevus, NY	9.1 (0.8)	1.5 (0.2)	23.5	0.145 (0.071)	0.126 (0.055)
Ralston, PA	15.5 (1.4)	1.5 (0.2)	29.4	0.150 (0.061)	0.147 (0.057)
Mean	24.04	1.84	34.12	0.142	0.138
A. asplenioides					
Shirley, NJ	28.2 (2.0)	2.0 (0.3)	35.3	0.127 (0.041)	0.127 (0.040)
Mountjoy Store, VA	22.8 (2.1)	2.2 (0.3)	41.2	0.133 (0.052)	0.123 (0.042)
Hopewell, VA	23.7 (1.8)	1.9 (0.2)	47.1	0.125 (0.038)	0.118 (0.036)
Sandy Run Swamp, NC	28.9 (1.5)	2.0 (0.2)	35.3	0.104 (0.039)	0.117 (0.041)
Pond Drain, VA	44.5 (2.3)	2.4 (0.3)	35.3	0.118 (0.049)	0.112 (0.046)
Mean	29.62	2.1	38.84	0.121	0.119
Mean across all populations	26.83	1.97	36.48	0.131	0.129

[a] A locus is considered polymorphic if the frequency of the most common allele does not exceed 0.95.
[b] Unbiased estimate (Nei, 1978).

TABLE 5. Values of the fixation index F as a measure of the conformance of population genotype ratios to those expected under Hardy-Weinberg equilibrium. F was computed for each polymorphic locus in each population, and tested for statistical difference from 0 (i.e. conformance to Hardy-Weinberg expectations) using the chi-square test. Pooling was carried out for loci with more than two alleles and therefore more than three genotypic classes. Chi-square tests were considered valid only if two of the three genotypic classes were represented by an expected values ⩾ 5. Results from non-valid tests are provided in brackets. Values marked as "ns" represent loci found to confirm to Hardy-Weinberg expected proportions ($p \geqslant 0.05$), and therefore are not statistically different from 0. Values marked with asterisks are statistically different from 0 at probabilities $p \leqslant 0.05$ (one asterisk), $p \leqslant 0.01$ (two asterisks), or $p \leqslant 0.001$ (three asterisks).

Locus	Mt. Ste. Hilaire QE	Barnet VT	N Hudson NY	Schenevus NY	Ralston PA	Shirley NJ	Mountjoy Store VA	Hopewell VA	Sandy Run Swamp NC	Pond Drain VA	
Ald										[−0.025 ns]	
Got		−0.040 ns				[−0.048 ns]		−0.091 ns		[−0.015 ns]	
Hk	[−0.042 ns]	[−0.028 ns]		[−0.048 ns]	[0.403 ns]	−0.124 ns	[−0.026 ns]	[−0.026 ns]	0.266 ns	[−0.039 ns]	
Idh	−0.007 ns	0.102 ns	0.148 ns	[0.267 ns]	−0.099 ns	−0.208 ns	[0.653***]	−0.163 ns	0.002 ns	−0.070 ns	
Lap	[10.132 ns]	−0.087 ns	0.216 ns		0.154 ns		[−0.043 ns]		[0.216***]	[−0.046 ns]	
Mdh-1	[−0.020 ns]	0.376**	[−0.061 ns]			[−0.065 ns]	[−0.034 ns]		[−0.032 ns]		
Mdh-2		[−0.010 ns]					[−0.071 ns]	[−0.053 ns]			
Mdh-3						[−0.015 ns]	[−0.034 ns]		[0.650***]		
Mdh-4	[1.000***]	0.192 ns	[−0.040 ns]				[−0.034 ns]	[−0.053 ns]			
Pgi-2	−0.375*	0.021 ns	−0.190 ns	[−0.266 ns]	−0.212 ns	−0.054 ns	0.136 ns	−0.156 ns	−0.088 ns	−0.048 ns	
Pgm-2	0.240 ns	0.120 ns	[−0.103 ns]		[−0.026 ns]	−0.167 ns	−0.280 ns	0.028 ns	0.259 ns	−0.057 ns	
6Pgd-1						[1.000***]					
6Pgd-2						[−0.015 ns]	[−0.040 ns]	[−0.026 ns]	[−0.017 ns]	−0.038 ns	
Skdh	[−0.020 ns]	−0.039 ns	[−0.061 ns]	[−0.048 ns]				[−0.071 ns]		[−0.010 ns]	
No. tests showing conformance to HW[1]	3[5]	8[2]	4[5]	0[5]	3[3]	5[5]	2[7]	5[5]	5[3]	5[5]	Total: 40[45]
No. of tests showing non-conformance to HW[1]	1[1]	1[0]	0[0]	0[1]	0[0]	0[1]	0[1]	0[0]	0[2]	0[0]	Total: 2[6]

[1] number of non-valid tests in brackets.

TABLE 6. F statistic analysis (Wright, 1965, 1978), including hierarchical analysis (Wright, 1978), for 16 polymorphic loci across *Athyrium filix-femina* populations in eastern North America. Statistical difference of F_{ST} from 0 was evaluated using contingency chi-square analysis. For hierarchical analysis, *Athyrium* populations were assigned to their respective taxa (*angustum* or *asplenioides*). Differences between values of F_{ST} and $F_{locality/total}$ are attributable to differences in computational method (discussed in Swofford and Selander, 1981).

	F Statistics			Hierarchical F-statistic Analysis (variance components in parentheses)			
Locus	F_{IS}	F_{IT}	F_{ST}	$F_{Locality/Total}$	$F_{Locality/Taxon}$	$F_{Taxon/Total}$	Total Limiting Variance
Ald	−0.025	−0.002	0.022 ns	0.012 (0.00007)	0.014 (0.00009)	−0.002 (−0.00002)	0.00623
Got	−0.060	−0.017	0.041 ns	0.028 (0.00109)	0.030 (0.00116)	−0.002 (−0.00007)	0.03895
Hk	0.116	0.160	0.049***	0.021 (0.00273)	0.031 (0.00398)	−0.010 (−0.00125)	0.12816
Idh-1	0.043	0.485	0.460***	0.452 (0.29124)	0.002 (0.00073)	0.451 (0.29050)	0.64377
Lap	0.077	0.204	0.137***	0.113 (0.02383)	0.092 (0.01886)	0.024 (0.00497)	0.21012
Mdh-1	0.036	0.060	0.025 ns	0.008 (0.00037)	0.014 (0.00068)	−0.006 (−0.00031)	0.04939
Mdh-2	−0.059	−0.013	0.044**	0.017 (0.00042)	0.016 (0.00040)	0.001 (0.00001)	0.02501
Mdh-3	0.302	0.321	0.028 ns	0.007 (0.00013)	0.002 (0.0003)	0.005 (0.00010)	0.01889
Mdh-4	0.208	0.230	0.029 ns	0.010 (0.00046)	0.014 (0.00067)	−0.005 (−0.00021)	0.04724
Pgi-2	−0.143	−0.012	0.115***	0.096 (0.03914)	0.040 (0.01551)	0.058 (0.02363)	0.40775
Pgm-2	−0.001	0.469	0.468***	0.459 (0.23433)	0.045 (0.01293)	0.434 (0.22140)	0.51042
6Pgd-1	1.000	1.000	0.039 ns	0.018 (0.00016)	0.023 (0.00020)	−0.004 (−0.00004)	0.00866
6Pgd-2	−0.032	−0.013	0.019 ns	0.011 (0.00037)	0.000 (0.00000)	0.011 (0.00037)	0.03325
Skdh	−0.052	−0.019	0.031***	0.013 (0.00061)	0.011 (0.00054)	0.001 (0.00006)	0.04782
Tpi-1	−0.070	−0.020	0.047 ns	−0.003 (−0.00014)	0.000 (0.00001)	−0.004 (−0.00015)	0.04269
Tpi-2	−0.214	−0.021	0.159***	0.137 (0.08853)	0.013 (0.00763)	0.125 (0.08091)	0.64843
Combined across loci	−0.052	0.217	0.255***	0.238 (0.68333)	0.028 (0.06343)	0.216 (0.601990)	

taxa with respect to the total (F_{XY}=0.216) was an order of magnitude greater than variance among populations within taxa (F_{XY}=0.028). Thus, differences between taxa explained most of the variance among populations with respect to the total (F_{XY}=0.238). This result is consistent with and explained by the large allele frequency differences at the four most polymorphic loci (*Idh-1, Pgm-2, Pgi-2*, and *Tpi-2*) between populations of different taxa as opposed to populations of the same taxon.

Values for Nei's Genetic Identity, I, (Nei, 1978) and Rogers' Genetic Similarity, S, (Rogers, 1972) were computed for each pair of populations (Tables 7 and 8). Values for these indices were consistently higher between populations of the same taxon, ranging from I=0.990 to 1.000 and S=0.930 to 0.975, than between populations of different taxa, ranging from I=0.875 to 0.938 and S=0.803 to 0.881 (Table 8).

Populations were clustered using the Unweighted Pair-group Method with Averaging (UPGMA) based on both S and I. The two indices resulted in very similar dendrograms that differed only in the association among some of the *A. asplenioides* populations; only the dendrogram based on S is illustrated (Fig. 5). Two clusters, each comprising all the populations of one taxon, were joined at S=0.849. Within the *A. angustum* cluster, the two northernmost populations Mt. Ste. Hilaire, QUE and Barnet, VT were placed as most similar, joined at S=0.975, and to this cluster the other three *A. angustum* populations were joined successively in order from north to south; the southernmost *A. angustum* population Ralston-1, PA joined the *A. angustum* cluster at S=0.938. The topology of this *A. angustum* cluster was identical in the dendrogram based on I (not shown). In *A. asplenioides*, the most similar populations based on S=0.966 were the southernmost population Sandy Run Swamp, NC and the next most southeastern occurring population Mountjoy Store, VA, and to these were joined the Pond Drain, VA population from the mountains of southwestern Virginia at S=0.958 to form a subcluster. A second subcluster, comprising the two northernmost *A. asplenioides* populations Shirley, NJ and Hopewell, VA, joined the more southern subcluster at S=0.947. The topology of the *A. asplenioides* cluster differed somewhat in the dendrogram based on I (not shown) indicating that the geographic "signal" in the *A. asplenioides* data is weaker than in the *A. angustum* data.

Discussion

The *A. filix-femina* complex, distributed across four continents and comprising as many as four North American taxa with overlapping ranges, provides an especially suitable context for exploring patterns and processes of divergent evolution and its taxonomic consequences in ferns. The two eastern North American taxa, *A. angustum* and *A. asplenioides*, long have been perceived as close relatives separable by distinctive characters that are consistent within the vast northern and southern areas they respectively occupy,

TABLE 7. Matrix of pairwise values for Rogers (1972) genetic similarity (above diagonal) and Nei (1978) unbiased genetic identity (below diagonal).

	Population									
	1	2	3	4	5	6	7	8	9	10
1. Mt. Ste. Hilaire, QE	-	0.975	0.962	0.953	0.939	0.853	0.865	0.847	0.862	0.881
2. Barnet, VT	1.000	-	0.956	0.938	0.930	0.851	0.864	0.853	0.858	0.879
3. North Hudson, NY	0.999	0.997	-	0.944	0.943	0.844	0.856	0.840	0.852	0.873
4. Schenevus, NY	1.000	0.996	0.995	-	0.942	0.841	0.842	0.831	0.843	0.863
5. Ralston, PA	0.993	0.990	0.994	0.993	-	0.817	0.821	0.803	0.825	0.843
6. Shirley, NJ	0.915	0.913	0.906	0.900	0.883	-	0.943	0.951	0.959	0.932
7. Mountjoy Store, VA	0.924	0.921	0.916	0.908	0.892	0.996	-	0.950	0.966	0.959
8. Hopewell, VA	0.912	0.911	0.903	0.893	0.875	0.998	0.997	-	0.951	0.937
9. Sandy Run Swamp, VA	0.924	0.921	0.916	0.905	0.891	0.998	1.000	0.998	-	0.956
10. Pond Drain, VA	0.938	0.936	0.934	0.923	0.911	0.990	0.998	0.990	0.996	-

but that intergrade and recombine to form a hybrid zone in their relatively narrow region of overlap. This perception is supported by the data from the present study. The spores of the two taxa show striking and consistent differences in the perispore sculpturing, a low papillate perispore in *A. angustum* versus a rugose perispore in *A. asplenioides*, and frequencies of allozymes exhibit strong differences between as compared to within the taxa. An abrupt shift in allele frequencies at the four most polymorphic loci (*Idh-1, Pgm-2, Tpi-2*, and *Pgi-2*) of *Athyrium* corresponds to the geographic boundary between *A. angustum* and *A. asplenioides*, i.e., between northern Pennsylvania and southern New Jersey in our sample. Although virtually all alleles were shared between the two taxa, some alleles that predominated or were frequent in one taxon were nearly absent in the other, e.g., $Idh\text{-}1^A$ of *A. asplenioides*, $Idh\text{-}1^C$ of *A. angustum*, and $Pgi\text{-}2^G$ of *A. angustum*. In other cases, alleles were more extensively shared between the taxa but at very different frequencies, e.g. $Idh\text{-}1^B$, $Pgm\text{-}2^B$, $Pgm\text{-}2^C$, and $Tpi\text{-}2^B$ (Table 3). UPGMA analysis of *Athyrium* resulted in two distinct taxon clusters joined at a substantially lower similarity value (S=0.849) than that joining populations within their respective taxon clusters (S=0.938 for *A. angustum*;

TABLE 8. Means of pairwise values for Rogers' Similarity (S) and Nei's Genetic Identity (I) for comparison within and between *Athyrium* taxa. Ranges are given in parentheses. Each category was represented by ten pairwise comparisons.

Taxon combination	I	S
angustum–angustum	0.996 (0.990–1.000)	0.948 (0.930–0.975)
asplenioides–asplenioides	0.996 (0.990–1.000)	0.951 (0.932–0.966)
angustum–asplenioides	0.911 (0.875–0.938)	0.848 (0.803–0.881)

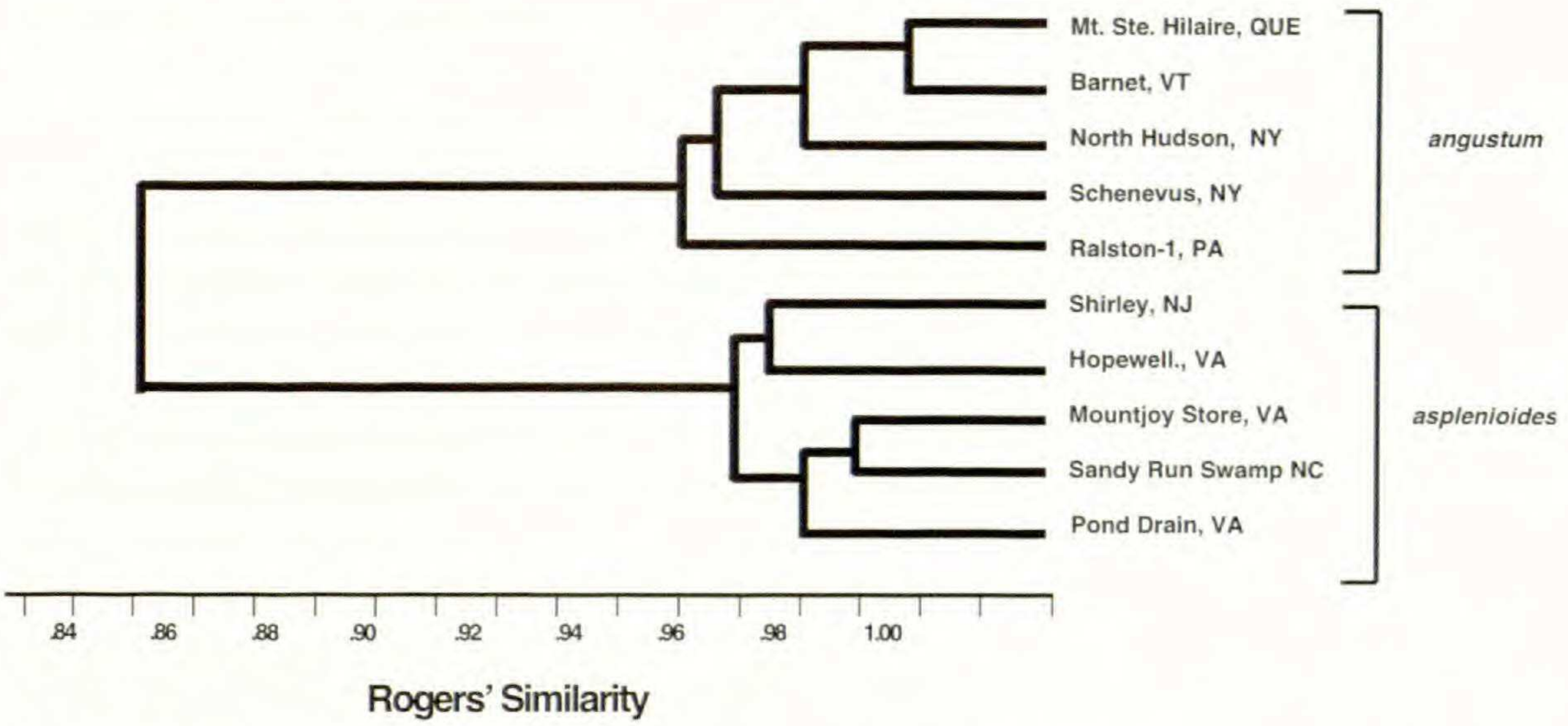

FIG. 5. Dendrogram resulting from UPGMA analysis based on pairwise values of Rogers' Similarity (Table 7).

S=0.947, for *A. asplenioides*). These spore and allozyme differences for the most part are consistent among populations of each taxon, yet there is evidence of introgressive hybridization in their region of overlap as discussed below.

The distinctness of these taxa and the consistency of the character differences specified by Butters (1917) frequently have been questioned by statements indicating that the taxa intergrade extensively (e.g., Benedict, 1934; Weatherby, 1936; Fernald, 1946; Shaver, 1954; Wherry, 1961). However, such statements seem anecdotal in that they are not accompanied by documentation of character combinations in specimens. The most extensive specimen-based data set is that of Liew (1971, 1972), who carried out a phenetic analysis of North American *Athyrium sensu lato* based on 170 specimens scored for 99 characters. Liew indicated that cluster analysis based on paired similarity coefficients separated each *Athyrium* taxon, including *A. angustum* and *A. asplenioides*, into its own cluster, but the summary dendrograms presented leave it uncertain as to whether some of the specimen placements were inappropriate or equivocal.

In the present study, the degree of differentiation between *A. angustum* and *A. asplenioides* may appear exaggerated by the omission of localities where the two taxa co-occur. This omission was neither intentional nor an oversight, rather it resulted from a failure to discover such localities despite a substantial effort to do so. A search for co-occurring populations in southern Pennsylvania resulted in finding only a few isolated individuals of *A. asplenioides* in this highly agriculturalized and urbanized region, and no sizable populations from which allele frequency data could be obtained. All individuals in populations sampled for the present investigation from Quebec south to northern Pennsylvania were readily assignable to *A. angustum* on the basis of leaf and spore morphology. Similarly, all individuals in

populations from North Carolina north to northern Virginia were assignable to *A. asplenioides*. However, a genetic influence from *A. angustum* is suggested by the high frequency of characteristically *A. angustum* alleles $Idh\text{-}1^B$ and $Pgi\text{-}2^G$ in the northernmost *A. asplenioides* population Shirley, NJ, as well as $Pgm\text{-}2^B$ and $Tpi\text{-}2^B$ in the highest elevation *A. asplenioides* population Pond Drain, VA. Evidence of introgressive hybridization is augmented by the intermediate spore morphologies encountered at the Shirley, NJ, locality. No individuals assignable to *A. angustum* were found at this site, but it is possible that *A. angustum* spores could have migrated from populations further north and effected hybridization (Wagner, 1943). The prevalence of fully intermediate spores in six of the individuals, five of which are heterozygous for taxon marker allozymes, suggest that these are first-generation hybrids. The skewed array of spore morphologies of other individuals suggests that they are backcrosses and provides evidence that hybridization has gone beyond the first generation resulting in introgression. Thus, spore and isozyme data combine to support previous morphology-based suggestions that the region of overlap between the taxa, and possibly higher elevations in the southern Appalachians as well, could represent a hybrid zone resulting from secondary contact between recently diverged sister taxa (Benedict, 1934; Shaver, 1954; Sciarretta *et al.*, ms).

Narrow hybrid zones in which formation of fertile hybrids and backcrosses result in intergradation between divergent taxa occur between numerous species or subspecies pairs of animals and angiosperms (reviewed by Arnold, 1997), and occur in a few agamosporous ferns (Gastony and Windham, 1989). Hybridization between fern species usually results in spore abortion due to abnormal meiosis, and introgressive hybridization between homoploid taxa as divergent as *A. angustum* and *A. asplenioides* is decidedly rare in ferns, with only three cases having been documented previously: (1) swarms of fully fertile hybrids involving *Pteris quadriaurita* and *P. multiaurita*, first generation as well as backcrosses, occur in disturbed forests of Sri-Lanka (Walker, 1958); (2) extensive hybridization among three species of the tree fern genus *Alsophila* resulted in a complex swarm of fertile hybrids in Puerto Rico, a scenario hypothesized to give rise to new species through allogamous allohomoploidy (Conant and Cooper-Driver, 1980; Conant, 1990); (3) morphological and molecular data provide evidence of hybrid swarms between *Polystichum munitum* and *P. imbricans* of northwestern North America (Mayer and Mesler, 1993; Mulleniex *et al.*, 1998). Of these, the situation in *Polystichum* most closely parallels that of the *Athyrium* taxa *angustum* and *asplenioides*. The two *Polystichum* taxa share alleles at most enzyme loci, but are differentiated with respect to frequencies at three loci resulting in a lower magnitude of genetic identity between the taxa (I= 0.842) than within each taxon (means: I=0.974 for *P. imbricans*, I=0.957 for *P. munitum*; Soltis *et al.*, 1990, 1991). Additionally, the *Polystichum* taxa hybridize introgressively, although they do not form a hybrid zone as in *Athyrium*; rather hybridization occurs at various localities across a broad area of sympatry between the two taxa (Mayer and Mesler, 1993; Mulleniex *et al.*,

1998). Maintenance of distinction between these two *Polystichum* species most likely results from a combination of their partial intersterility and diversifying selection imposed by the very different habitats—forests versus exposed cliffs—occupied by *P. munitum* and *P. imbricans* respectively.

The nature, frequency, and geographic extent of hybridization between *A. angustum* and *A. asplenioides* remain uncertain and merit further research that combines field, herbarium, molecular, and breeding studies. It is critical to determine with greater precision and full documentation the degree to which these two taxa maintain versus blend their macromorphological, micromorphological, and allozymic differences where they coexist. It is unknown whether there is preferential mating within taxa, whether taxon characters tend to remain associated in the face of hybridization or are completely recombined, and whether there exists a cline for allozyme frequencies and morphological characters within the overlap region. There is a need for intensive exploration for coexisting *A. angustum* and *A. asplenioides* populations in the areas of their overlap from the eastern seaboard through the midwest, and in the southern Appalachians where the existence of cryptic taxa has been hypothesized (Wagner and Wagner, 1966). Isozyme studies of these populations should be coordinated with critical analyses of morphological character combinations obtained from numerous specimens and with experimental crosses that can quantify the propensity of the taxa to hybridize.

Beyond the taxonomic significance of hybridization, the unprecedented formation of normal intermediates between spores of such divergent morphology provides an opportunity to gain insight into the genetics underlying spore morphology (Schneller, 1989). The variability of spore morphology within individuals of the putative primary hybrids from the Shirley, NJ site, which includes expression of parental types, indicates that inheritance is polygenic rather than a simple one-or-two-gene inheritance mechanism, and that the spore genotype determines or at least influences perispore phenotype. Contrasting observations and inferences were obtained by Schneller (1989), who reported the formation of normal intermediate spores in experimentally produced hybrids between *A. angustum* and *A. asplenioides*, but found that all spores from a sporangium were of the same type, implicating sporophytic determination of the perispore morphology. Explanations that can account for these differing observations include the possibility that the Shirley, NJ, plants were not true F1 hybrids, or that there may be a maternal effect that varies as a polymorphism. Clearly, further studies of the inheritance of spore morphology are in order.

TAXONOMIC CONCLUSION: AT WHAT RANK SHOULD *A. ANGUSTUM* AND *A. ASPLENIOIDES* BE RECOGNIZED?—Over the second half of this century, the nature of pteridophyte species has been clarified significantly by application of technological advances in cytology and molecular systematics in combination with detailed morphological and field studies (Manton, 1950; Wagner, 1963; Haufler, 1987, 1989; Paris *et al.*, 1989; Conant, 1990). Nonetheless, definitive ranking of closely related divergent taxa with overlapping ranges, such as

the two *Athyrium* taxa considered here, remains challenging due to the unsettled controversy as to the nature and definition of species (e.g., Mayr, 1992; Davis and Nixon, 1997; Baum, 1998; DeQueroz, 1998) as well as to the unpredictable nature of reproductive interactions between diverged taxa experiencing secondary contact (Arnold, 1997). The rank assigned to *A. angustum* and *A. asplenioides* has varied considerably in floristic treatments published in this century. While Small (1938) and Wherry (1948, 1961) followed Butters (1917) in treating these taxa as separate species, other authors have tended to treat them as infraspecific taxa, either subspecies (Lellinger, 1985) or varieties (Fernald, 1950; Mickel, 1979; Cody and Britton, 1989; Gleason and Cronquist, 1991; Kato, 1993).

Spore and isozyme data combined indicate that populations of these two taxa are significantly divergent, exhibiting greater differences than ordinarily encountered within single species of ferns thus far studied. However, the fertility of hybrids (Schneller, 1989) provides a potential for introgression between the two taxa. The preliminary evidence that hybridization and introgression do in fact occur indicates that *A. angustum* and *A. asplenioides* would be considered conspecific under species definitions as different as the Biological Species Concept (Mayr, 1942) and the Phylogenetic/Diagnostic Species Concept (Cracraft, 1983; Davis and Nixon, 1992). Nonetheless, in practice numerous pairs of taxa that form hybrid swarms or zones are treated as distinct species (Grant, 1981; Arnold, 1997).

The occurrence of northern and southern infraspecific taxa in eastern North American *Athyrium* is paralleled in *Pteridium aquilinum* L., which comprises northern and southern varieties *latiusculum* (Desv.) Underw. and *pseudocaudatum* (Clute) Heller, respectively, and which shows north to south clines in allele frequencies near the overlapping taxon boundary (Speer *et al.*, 1998). However, the pattern of allozyme variation in *Pteridium* differs from that in *Athyrium* in that the most abrupt shift in allele frequencies occurs within the range of *P. latiusculum*, genetic identities between populations of the two *Pteridium* varieties ranged substantially higher (I= 0.916 to 0.999) than those of the two *Athyrium* taxa (I=0.875—0.938, mean =0.911), and UPGMA clustering failed to separate the varieties (Speer *et al.*, 1998). On the basis of the lack of genetic differentiation between *P. latiusculum* and *P. pseudocaudatum*, variety was indicated as the highest rank at which to recognize them (Speer *et al.*, 1998). In contrast, the more substantial differentiation between the *Athyrium* taxa *angustum* and *asplenioides* and the consistent characters uniting a vast number of individuals north and south of their hybrid zone suggest that the taxa should be ranked at least at the level of subspecies. Ranking at the species level would not be inconsistent with the treatment of such taxa in the broader plant literature.

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